







Antibacterial and Solubility Analysis of Experimental Phytotherapeutic Paste for Endodontic Treatment of Primary Teeth

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Academic Editor: Catarina Ribeiro Barros de Alencar

Received: 05 February 2020 / **Review:** 17 May 2020 / **Accepted:** 21 July 2020

How to cite this article: Freire AR, Freire DEWG, Sousa SA, Serpa EM, Almeida LFD, Cavalcanti YW. Antibacterial and solubility analysis of experimental phytotherapeutic paste for endodontic treatment of primary teeth. *Pesqui Bras Odontopediatria Clín Integr.* 2021; 21:e0007. <https://doi.org/10.1590/pboci.2021.005>

ABSTRACT

Objective: To evaluate the antibacterial effect and the solubility of experimental root canal filling pastes containing the phytoconstituents terpineol and cinnamaldehyde. **Material and Methods:** Minimum Inhibitory Concentration (MIC) of each phytoconstituent was determined against *Enterococcus faecalis*. Five groups of antibiotic pastes based on zinc oxide were obtained by mixing: only terpineol, only cinnamaldehyde, terpineol and cinnamaldehyde combined, chlorhexidine (antibiotic control), and CTZ paste (control paste). Antibacterial activity was analyzed through direct contact test within 24 and 72 hours. Solubility was evaluated by spectrophotometry within 48 and 144 hours. Antibacterial activity data were analyzed descriptively, and solubility data was analyzed using ANOVA and Tukey tests ($p < 0.05$). **Results:** The MIC obtained for terpineol and cinnamaldehyde were, respectively, 2000 $\mu\text{g}/\text{mL}$ and 500 $\mu\text{g}/\text{mL}$. After 24h, only the terpineol paste did not inhibit *E. faecalis* growth. After 72h, all groups inhibited *E. faecalis* growth. After 48h, the highest solubility was verified in the terpineol paste ($p < 0.05$), and no differences were detected among other groups ($p > 0.05$). After and 144h, highest solubility was observed in the terpineol paste ($p < 0.05$), followed by the CTZ paste ($p < 0.05$). No differences were detected for cinnamaldehyde, terpineol+cinnamaldehyde and chlorhexidine pastes ($p > 0.05$). **Conclusion:** Pastes containing cinnamaldehyde or terpineol+cinnamaldehyde showed antibacterial activity against *E. faecalis* similar to CTZ paste, with lower solubility.

Keywords: Root Canal Filling Materials; Tooth, Deciduous; Plant Extracts; *Enterococcus faecalis*.

Introduction

Endodontic treatment of primary teeth presents particularities related to the complex anatomy and topography of its root canal system, showing sharp curvatures and several accessory channels [1]. Such characteristics make instrumentation hard and require the use of strategies to reduce or eliminate the remaining microorganisms. Therefore, antimicrobial sealing pastes' efficacy is an important aspect of post-treatment success [2,3].

In addition to the necessary antibacterial activity, several other properties are desirable to endodontic pastes, such as biocompatibility, radiopacity, no tooth discoloration or aggression to the successor permanent tooth germ, degradation similar to the physiological resorption process of the deciduous tooth, having low solubility in water, among others [4].

Among the root canal filling pastes indicated in literature, CTZ (chloramphenicol, tetracycline, zinc oxide and eugenol) presents satisfactory clinical results, as well as a simple and easy-to-perform technique [3,5,6]. However, this material is still not widely used, due to factors such as the fear of professionals regarding biocompatibility of the paste (a few numbers of studies analysed this propriety) [7] and the lack of studies that verified long-term clinical success of this therapy [3].

These challenges are necessary to search for new therapeutic products with antimicrobial activity capable of representing innovative and safe endodontic treatment alternatives for primary teeth. Under this perspective, it is possible to consider phytoconstituents' use because they present relevant biological properties against many microorganisms involved in endodontic infections [8-10].

Terpineol and cinnamaldehyde are phytoconstituents that show an important antimicrobial action [11,12]. Terpineol (terpinen-4-ol) is the main constituent of the essential oil TTO (tea tree oil) distilled from *Melaleuca alternifolia* [11]. Cinnamaldehyde (trans-cinnamaldehyde) is obtained from *Cinnamomum zeylandicum*, present in the cinnamon bark extract [13]. The use of terpineol and cinnamaldehyde in constitution of root canal filling pastes was not studied before, although these substances have therapeutic potential and vast parameters to be explored and tested.

Thus, this study aimed to evaluate the antibacterial effect and the solubility of experimental root canal filling pastes containing the phytoconstituents terpineol and cinnamaldehyde and compare them with the CTZ paste, frequently used in endodontic treatment of primary teeth.

Material and Methods

Microorganisms Used

Considering the major role of *Enterococcus faecalis* in endodontic infections, this study used the reference strain *Enterococcus faecalis* (ATCC 29212), obtained from the FIOCRUZ's collection of reference microorganisms. The strain was maintained in Brain Heart Infusion (BHI) culture medium enriched with 5% defibrinated blood (blood agar). Standardized solutions of microorganisms were used at a concentration of 1×10^8 CFU/mL determined in a spectrophotometer, at an absorbance of 0.1, under 600 nm wavelength. The concentration of microorganisms established for the Minimum Inhibitory Concentration (MIC) assays was 1×10^5 CFU/mL, which was obtained by the 1000× dilution of the previously standardized inoculum.

Determination of Minimum Inhibitory Concentration (MIC) of Phytoconstituents

The MIC (lower concentration of substances capable of inhibiting the visible growth of the evaluated microorganisms) was determined according to the M7-A9 standardization of the Clinical Laboratory and

Standards Institute (CLSI) [14], using the broth microdilution technique, developed in three independent experiments. Wells from a 96-well culture plate were filled with 100 μL of the RPMI 1640 culture medium. Then, 100 μL of phytochemical working solutions with concentration 8 mg/mL (8000 $\mu\text{g}/\text{mL}$) were inserted into the first row wells of the microdilution plate. Subsequently, the serial dilution of the compounds was performed by transferring 100 μL of the most concentrated well to the least concentrated, so the substances passed through serial dilutions of 1:2 (v/v). 100 μL of *Enterococcus faecalis* bacterial inoculum (ATCC 29212) at 1×10^5 CFU/mL was inserted into all wells. The samples were incubated at 37°C for 24 hours in microaerophilic. Viability control of microorganisms and sterility control of culture mediums were performed to guarantee accuracy of the method. The results were analyzed visually, considering the inhibition of microbial growth at the bottom of the wells.

Constitution of Antibiotic Pastes

The phytoconstituents terpineol (ref. 432628, SigmaAldrich, St. Louis, MO, USA) and cinnamaldehyde (ref. W228613, SigmaAldrich, St. Louis, MO, USA) were obtained in their pure form. Technical report of the substances, molecular weight, purity, density and other characteristics can be assessed in <http://www.sigmaaldrich.com/brazil>. The phytoconstituents and controls (0.1 mL) were mixed by spatulation to 1 g zinc oxide powder p.a. (Biodinâmica Química e Farmacêutica LTDA, Ibioporã, PR, Brazil) until saturated, in order to obtain a paste-like consistency compatible with the material used as an intracanal medication of deciduous teeth. Four types of experimental root canal filling pastes were obtained: terpineol + zinc oxide, cinnamaldehyde + zinc oxide, terpineol + cinnamaldehyde + zinc oxide, chlorhexidine + zinc oxide. CTZ paste (500 mg of chloramphenicol, 500 mg of tetracycline and 1000 mg of zinc oxide, micronized using mortar and pestle, and saturated in 1 drop of eugenol) was used as the standard for comparison.

Evaluation of the Antibacterial Activity of Pastes

The antibacterial effect of the pastes was evaluated by the direct contact test [15]. Test specimens ($n=5/\text{group}$) [16,17] were immersed in 2 ml of culture medium containing *E. faecalis* bacterial inoculum (ATCC 29212) at 1×10^8 CFU/ml concentration. After 24 and 72 hours, samples (10 μL) of the culture medium inoculated by microorganisms were seeded using the Muller Hinton agar plates to verify the microorganisms' viability. The plates were incubated at 37°C for 24 h, and after that period, the number of viable microorganisms was quantified.

Evaluation of the Solubility of Pastes

Test specimens from the test groups ($n=5$) were packed in plastic matrices, obtained from 10 μL sterile bacteriological loops. The specimens were inserted into wells of a 24-well culture dish, and in each of them was pipetted 1 ml of distilled water. The plate was kept in an oven at 37°C. The solubility of pastes was visually identified by the observation of residues in the containers and by the turbidity of the distilled water, verified in a spectrophotometer, at the wavelength of 600 nm [18-20].

Data Analysis

Data were tabulated and statistically analyzed in the Statistical Package for Social Sciences program (SPSS, v. 21, IBM, Chicago, IL, USA). The data obtained from the CIM and Direct Contact tests were analyzed

descriptively, using means and standard deviation. The absorbance data passes obtained for the solubility test were evaluated by Analysis of Variance (ANOVA) by two factors (group and time), with Tukey post-hoc test, considering $p < 0.05$ and statistical power of 80%. Kolmogorov-Smirnov test was used to confirm a normal distribution ($p > 0.05$). The null hypothesis was that experimental pastes containing phytoconstituents do not differ from CTZ paste (control) regarding to antibacterial activity and solubility..

Results

The MICs obtained against *Enterococcus faecalis* for terpineol and cinnamaldehyde are shown in Table 1. Both results coincided with the CBM (Minimum Bactericidal Concentration) of each phytoconstituent.

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) obtained for Terpineol, Cinnamaldehyde and Chlorhexidine.

Parameters	Substances		
	Terpineol	Cinnamaldehyde	Chlorhexidine
MIC	2000.0 µg / mL	500.0 µg / mL	7.8125 µg / mL
MBC	2000.0 µg / mL	500.0 µg / mL	7.8125 µg / mL

In the analysis of the antibacterial activity of antibiotic pastes against *E. faecalis*, only the terpineol paste could not inhibit the growth of the microorganism after 24 h. After 72 hours, all analyzed pastes showed areas of inhibition, free of viable microorganisms. The results of the direct contact test are given in Table 2.

Table 2. Viability of the suspension of *Enterococcus faecalis* after direct contact test, according to the groups studied.

Time	Groups				
	GCTZ	GT	GC	GTC	GCHX
24 h	0	+++	0	0	0
72 h	0	0	0	0	0

+++; Viable; 0; Not Viable.

Statistical analysis revealed that, within 48 h, only the terpineol paste differed from the others, and no statistically significant differences were observed among CTZ paste, cinnamaldehyde paste, terpineol+cinnamaldehyde paste and chlorhexidine paste (Table 3). After and 144 h, highest solubility was observed in the terpineol paste ($p < 0.05$), followed by the CTZ paste ($p < 0.05$). No differences were detected for cinnamaldehyde, terpineol+cinnamaldehyde and chlorhexidine pastes ($p > 0.05$) (Table 3).

Table 3. Means and standard deviations of the absorbance at 600 nm used to assess the different pastes' solubility after 48 and 144 hours.

Time	Groups				
	GCTZ	GT	GC	GTC	GCHX
48 h	0.1346 ± 0.0958	2.0 ± 0.0*	0.2334 ± 0.0994	0.1348 ± 0.0070	0.13625 ± 0.2125
144 h	0.4360 ± 0.2087*	2.0 ± 0.0*	0.1352 ± 0.1070	0.0562 ± 0.1450	0.1823 ± 0.0786

*Indicates a statistically significant difference in ANOVA test ($p < 0.05$) regarding the other groups.

As shown in Figure 1, the solubility test showed a clear difference in the coloration of the solutions between the studied groups. The presence of a yellowish tone in the solution containing the CTZ paste was observed. It was possible to verify the total solubilization of the terpineol paste.

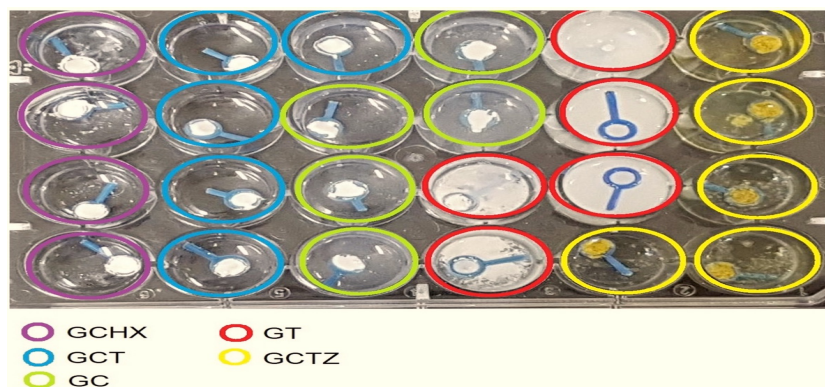


Figure 1. Solubility test of pastes after 48 h. There are qualitative differences in the coloration of the solutions, especially in the GT and GCTZ groups.

Discussion

This study demonstrated that the paste composed by phytoconstituents terpineol and cinnamaldehyde showed antibacterial performance similar to the CTZ paste. *E. faecalis* was chosen as the reference microorganism because it is frequently found in endodontic infections of deciduous teeth and presents mechanisms of virulence and resistance to antimicrobials [21]. Regarding solubility, the paste consisted of combined phytoconstituents showed better results than CTZ paste after a longer observation time, showing a lower solubility. These results suggest that further investigations are necessary to assess the efficacy and effectiveness of phytoconstituent-based endodontic pastes.

Terpineol and cinnamaldehyde had their efficacy proven against different human pathogens [11]. However, literature is scarce regarding studies that evaluated antimicrobial activity of these isolated phytoconstituents against *E. faecalis*. Some authors [8,9,22-25] found a potent antibacterial effect of essential oils containing such phytoconstituents in their composition. However, since essential oils are complex mixtures of several compounds with different properties, the antimicrobial efficacy found in these investigations can not be attributed specifically to terpineol or cinnamaldehyde.

The results obtained in this research confirm the antimicrobial activity of terpineol and cinnamaldehyde against *E. faecalis*. The MIC values obtained coincided with the Minimum Bactericidal Concentration (MBC), indicating that, at those concentrations, the phytoconstituents can inhibit the growth of the *E. faecalis* and also have a bactericidal action, leading to its destruction. This study corroborates the MIC and MBC values found for terpineol and cinnamaldehyde against *E. faecalis* and other gram-positive bacteria [26-28].

The experimental root canal filling paste composed by combined phytoconstituents showed a better antibacterial effect than the one consisted of only terpineol. Previous studies have shown a synergism between terpineol and cinnamaldehyde when interacting with other essential oil components [27,29]. However, the paste composed of isolated cinnamaldehyde presented similar results that consisted of a combination of terpineol and cinnamaldehyde. Therefore, further studies are necessary to understand the interaction between these phytoconstituents better, requiring longer periods of observation to verify if these effects remain constant over time.

Regarding solubility test, the paste composed of terpineol alone showed results that were not very encouraging in this respect, undergoing it to total solubilization within 48 h. In addition, CTZ also presented higher solubility within 144 h. CTZ elicited a yellowish coloration in the solution, that became even more







intense over time. This is probably due to the presence of tetracycline, which gives such coloration to the CTZ paste. The liberation of pigments by CTZ in aqueous medium may strengthen the theory described in the literature that the material represents a risk factor for color change in the successor permanent teeth, causing yellowish or brownish spots, due to the impregnation of tetracycline with dental tissues [30]. However, there is no evidence about this effect, and the occurrence of defects in the enamel of permanent teeth seems to be more related to the inflammatory process in the periapical region of deciduous tooth, due to the carious process [31,32].

The analysis of the results of this study showed that the paste composed by the combination of the two phytoconstituents presented good results in relation to the solubility and antibacterial activity against *E. faecalis*. Thus, its use in endodontic treatment of primary teeth would represent a viable alternative in a superficial analysis. However, this study has some limitations, such as the use of only one reference microorganism in planktonic form and the absence of simulation of conditions similar to those found in the oral environment. This is a preliminary study, and the application of this paste in the clinical practice of pediatric dentistry requires another in vitro and in vivo studies that considered several other variables, such as biocompatibility, stability, and security.

Conclusion

Experimental root canal filling pastes containing cinnamaldehyde or terpineol+cinnamaldehyde showed antibacterial activity against *E. faecalis* similar to CTZ paste, and lower solubility. Further studies must be carried out to analyze the viability and security of root canal filling pastes with terpineol and cinnamaldehyde.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.			

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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